AD_____

Award Number: W81XWH-06-1-0234

TITLE: Molecular Identification of Human Fungal Pathogens

PRINCIPAL INVESTIGATOR: Brian L. Wickes, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health Sciences Center
San Antonio, TX 78229-3900

REPORT DATE: March 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-03-2007	Annual	23 Feb 2006 – 22 Feb 2007

4. TITLE AND SUBTITLE

Molecular Identification of Human Fungal Pathogens

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0234

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Brian L. Wickes, Ph.D

Email: wickes@uthscsa.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Health Sciences Center
San Antonio, TX 78229-3900

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Each task that was proposed in the first 12 months of this study is on, or ahead of schedule. Methods have been standardized so that they can be performed and accomplished independent of the species of fungus. We have developed a simple universal extraction method and a universal PCR method, which most importantly, uses a single extraction reagent and the same set of primers for each isolate. Databases have been set up and data are now being added. A web portal has been created to allow access to search algorithms for the database. Preliminary identifications using our methods have been successful and resulted in a publication, as well as a new collaboration on an NIH grant. Type cultures and clinical cultures are being added to our collections, and their sequences are being added to the databases. The framework, from database entries to sequence recovery, has been established and will be continually ramped up as we add sequences at a faster rate. We will also continue to improve our methods, such as a filter based DNA extraction (for easy transport) and sequencing (for cost reduction), in order to make the entire process as efficient as possible.

15. SUBJECT TERMS
rDNA, SEQUENCING, PCR, BLAST

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION: This proposal will develop a biocurated database of DNA sequences that can be used for the identification of human fungal pathogens. Identification of non-routine fungi from clinical specimens cannot be reliably done without specific training in mycology. Unfortunately, individuals with this training are in short supply in both civilian and military hospitals. The objective of this study is to enable laboratory technicians to make proper identifications without experience in mycology by using standardized techniques developed in this proposal to generate a DNA sequence. This sequence can then be used to search an internet-accessible database. The output from this database will result in an identification that utilizes proper and consistent nomenclature, allowing technicians to provide an appropriate identification, which will allow clinicians to more efficiently select the proper treatment course. The significance of our study will be to enable any clinical laboratory, regardless of mycological expertise, to identify any human fungal pathogen faster and more accurately than is presently possible, using a single assay.

BODY: This reporting period is the first of this award and describes work completed or initiated on three of the four tasks. The three tasks described in this report were to be started in the first year at various times and completed by the first year (task 1 and all sub tasks), year and one half (task 2 and all sub tasks), or year 2 (task 3, sub task 1 only). Presently all tasks are on schedule, with some tasks ahead of schedule. There have been no changes to the original Statement of Work, and no major problems that have inhibited the proposal.

Progress report on Task 1. **CREATION OF AN INTERNET-ACCESSIBLE, SEQUENCE DATABASE FOR MOLECULAR IDENTIFICATION OF ALL KNOWN HUMAN FUNGAL PATHOGENS.** (Months 1-12)
The objective of this task was to develop a way to bring our database to the Internet, so that it would be accessible from anyplace that had computer access, including military hospitals or medical facilities. This objective had a number of components, which were divided into creation of the actual Internet portal (webpage), two databases of sequence and strain information (one located in our bioinformatics core to be used for the actual searches, and one on a desktop computer for data storage), and finally, the actually algorithms that allow sequence searching and manipulation. The final component consisted of the actual strain identification of cultures that represented the type culture of each species in the database, and back up cultures that would serve as additional sequences for the same species.

The web portal for our database has been designed and a domain at UTHSCSA has been acquired for hosting this web page. The web page provides all of the information about the Pathogenic Fungi rDNA Identification System (PFRIS), and has the links that take users directly to the search software**. Figure 1** displays the web page that will serve as the portal to our database.

Fig. 1. PFRIS Webpage portal. The figure displays the web page that will be used as the portal into the PFRIS database. Information about the database is shown in the left hand column with links to take users to additional information. The description is shown in bold blue, and the button that takes users to the search function is listed as "Proceed to PFRIS".

Embedded within the web page will be our searchable database, which has multiple fields that are attached to each type culture sequence (**Figure 2**). Included in this database will be additional tables that allow the entry of more detailed data. For example, under the field listed as ContributorID a sub field will open that will allow detailed information to be entered about the contributor (contact information, etc).

Data Entry Form;

Field	Type	Null	Key	Default	Extra
RecordID	int(11)		PRI	NULL	auto_increment
revision	int(11)			0	
PFRISnumber	int(11)			0	

EntryDate	datetime			0000-00-00 00:00:00
CollectionDate	date	YES		NULL
GenomeSequence	tinyint(4)			0
TypeStrain	tinyint(4)			0
TypeInfo	text	YES		NULL
TypePubl	text	YES		NULL
TeleomorphGenus	tinytext	YES		NULL
TeleomorphSpecies	tinytext	YES		NULL
AnamorphGenus	tinytext	YES		NULL
AnamorphSpecies	tinytext	YES		NULL
ClinicalName	tinytext	YES		NULL
SpecimenType	int(11)	YES		NULL
SpecimenSource	int(11)	YES		NULL
CultureID	int(11)	YES		NULL
CollectionID	int(11)	YES		NULL
ContributerID	int(11)	YES		NULL
dataReferences	text	YES		NULL
GenBankNum	tinytext	YES		NULL
PFRISsequence	text	YES		NULL
ITSsequence	text	YES		NULL
D1D2Sequence	text	YES		NULL

Fig. 2. Data entry form. The figure shows the fields that will be used for the PFRIS database. Where appropriate, fields will have sub fields to incorporate more information and allow relational interactions of data within each record.

Searches will be performed using the standard NCBI BLASTn program (1), which has been downloaded. This program can be modified, and has been re-written to change the output so that more information can be recovered from each search. Part of this modification will include alerts that will notify users that their “hit” has found an organism that may have some nomenclature issues associated with it. The alert function will be used whenever there is a current issue with an organism that clinicians or technicians may need to be aware of. While the most common issue will be nomenclature problems, we can use the alert flag to direct users to any pertinent out put field that comes up after the BLAST search. In addition to the BLAST program, we will also incorporate other algorithms into our search function, which will allow users to manipulate results. An example would be a program that presents output data as phylogenetic trees in order to show figuratively how their sequence of interest is related to the sequences of other species.

The second sub task of this aim is the creation of our master culture collection. This sub task is an ongoing task that will continue throughout the proposal. Through research of existing culture collections, our own culture collections, and the literature in Medline, we have cross-referenced all of the available isolates with isolates that are pathogenic for humans. These isolates are being tabulated to come up with a master list of fungi that are potential human fungal pathogens. Although pathogenesis is a complex definition, we used a general definition of a fungus being isolated from a human specimen, whether it is causing a clear disease or not. *In our opinion, this*

preliminary survey may result in a new paradigm for medical mycology because the number of pathogenic fungi could be grossly underestimated. All Medical Mycology reference books that we have seen cite 300-500 species of fungi being pathogenic for humans. **Our preliminary survey suggests this number could be 2-3x larger.** This observation may be one of the most important achievements of this proposal since it confers added significance to our sequencing hypothesis of fungal identification. While we continue searching sources for the most accurate number, we have been accumulating strains and sequencing these isolates. Presently we have approximately 300 sequences already (though the major sequencing effort won't begin until task 3). Importantly, we have made every effort to conserve funds by taking a number of approaches. First, we have utilized the USDA culture collection, which does not charge for isolates, but will only send 24 per year. Nonetheless, this will allow us to add 100 additional type cultures to our collection. Second, we have used all of the existing type cultures from our own database, and will not need to purchase these. Third, by comparing prices, we have found that the major fungal culture collection in Amsterdam is much less expensive than the American Type Culture Collection (ATCC) in the U.S. The only difficulty is filling out the import forms, which we have become proficient at since we have placed a number of orders. Lastly, we have been in contact with the ATCC and reminded them that their cultures are much more expensive than others, and that we would be willing to develop an agreement with them to provide comparative or free cultures to us in exchange for sequencing data. These negotiations are on going. Combined, these efforts will allow us to stretch our grant dollars as far as possible, which will be translated into more data.

In the last sub aim we have been formatting our database to insure proper fungal nomenclature is linked to each species. To do this accurately, we have stored all information associated with each isolate on a desktop computer using a commercial software database (Filemaker Pro). This database allows us to edit and reconfirm all data prior to entry into the public database. It also allows us to internally track each isolate and where sub cultures our stored.

Progress report on Task 2. **DEVELOPMENT OF STANDARDIZED PROTOCOLS FOR PCR AND SEQUENCING TEMPLATE PREPARATION.** (Months 6-18)

Because the major goal of this proposal was to develop a system that could be used by people with no formal training in mycology, a crucial step was to develop a simple, universal extraction procedure that could be applied to any fungus. A number of procedures were evaluated, which ranged from simple (fast & easy, but low yield) to complex (laborious & long, but high yield). One procedure was settled on due to its ease of preparation and high PCR success rate. This procedure uses a commercial reagent from Applied Biosystems called Prepman Ultra, and simply requires a suspension of cellular material in the solution, heating for 15 minutes, and removal of a few microliters of supernatant, after centrifugation, that serves as PCR template DNA. After standardizing growth conditions and amount of material to prepare, a study was conducted to measure extraction success (denoted by generation of a PCR product). Two

hundred sixty five yeast or filamentous fungi were prepared for PCR using this procedure. Table 1 shows the results;

Table #1. DNA isolation success rate.

Morphology	No. Preps	Succeeded	Failed	% Successful
Yeast	155	155	0	100
Hyphal	110	100	10	90.9
Total	265	255	10	96.2

The total success rate for this procedure was 96.2%, with 100% of yeast isolates yielding template DNA and 91% of filamentous (mould) isolates yielding DNA. If the extraction was repeated on the failed isolates one or more times, eventually a PCR product was generated. These results demonstrated that a DNA extraction protocol could be standardized that would yield template DNA from any species of fungus. Although this sub aim has been accomplished, additional experimentation is being performed to determine if even easier or more convenient extractions can be developed. We are particularly interested in a method that uses cards employed for blood specimens, which work by applying the specimen to a filter matrix, heating the card, and then washing. A portion of the card is removed by hand punch and placed directly in the PCR tube. This approach would be particularly useful for specimens that need to be shipped, since the isolates are rendered inviable by the process, and can be mailed in a simple envelope instead of a cumbersome biohazard container.

The second sub aim of this task has been completed. In this sub aim a universal PCR reaction was designed so that we would be able to amplify sequences from any fungus. The major aspect of this aim was to design universal primers and then develop a PCR mix, which would be able to reliably amplify template from any specimen, even pigmented isolates that contain PCR inhibitors. In order to design primers, we used the historic rDNA primers (2, 3) as a starting point and then downloaded the corresponding rDNA sequences, plus flanking regions, from the NCBI Entrez Genome Project site for fungal genomes. Contigs from approximately 50 fungal genomes were then searched with four primers most frequently used for amplifying fungal rDNA. The alignments revealed that the priming sites were 100% conserved, which confirmed that our choice of universal primers was correct. Importantly, this search included sequences from all four fungal phyla. We have used the four primers (ITS1, ITS4, NL1, NL4) for all of our PCR reactions and have found 100% amplification. Therefore, these four primers have been confirmed as being truly universal for amplification of fungal rDNA sequences.

Progress report on Task 3. **GENERATION OF TYPE SEQUENCES**. (Months 6-36)

During the course of developing optimized conditions for DNA extraction, we have begun to obtain sequences from both our type cultures as well as clinical isolates. The extraction protocol has proven diverse enough to work with all major fungal phyla, consequently, it has been incorporated into our standardized protocol. We have generated two hundred ninety three sequences, which include seventy-six sequences derived from type cultures. Forty-two sequences have been recovered from genome sequences at the NCBI site at the National Institutes of Health and one hundred seventy five have been obtained from clinical isolates. In addition to utilizing the DNA sequence for identification purposes, we have also investigated the length of each sequence and have been surprised by the data. The sequence length varies from 884 bp for *Dipodascus capitatus* to 1695 bp for *Pythium insidiosum*. Investigation of sequences within species shows slight variation of size over a few base pairs, so sequence length is not definitive for identification, but it has proven to be informative. At the end of the study this characteristic will be investigated through additional bioinformatics.

In order to hasten the sequencing process and allow us to generate more sequences, we have made a slight modification to the PCR protocol. In the original proposal, we planned to PCR and then clone each amplicon. However, we have found that this step is not necessary since PCR products can be sequenced directly. We clone PCR products that prove to be problematic to sequence due to secondary structure, or yield products that are unusually long. In some cases secondary primer design to obtain sequence towards the end of a long read have been included. *Importantly, from DNA prep to PCR to DNA sequencing, we have not encountered any fungus from which a sequence could not be obtained.*

Lastly, in addition to beginning to add sequences from type cultures, we have started included sequences from culture collection isolates that we already have, as well as sequences from actual clinical isolates. In instances where sequences from clinical isolates have been used, the collaboration with members of the UTHSCSA Fungus Testing Laboratory (members listed in the proposal) has proven to be invaluable. We have been able to combine sequence identification with traditional morphological and biochemical identification to insure a high level of quality control for our database entries. This interaction also allows us direct access to fresh clinical isolates, and has allowed us to identify and add many unusual isolates from patients within as well as outside of the US. Finally, as our protocols have become standardized, we have been able to add sequences at a faster rate, which has allowed us to focus on cost efficiency due to repetition. One area in which we have already made improvements is in the sequencing reactions themselves. Typical reactions use 1/4 strength sequencing reagent (Big Dye cycle sequencing kit). We have reduced this level to 1/16 and are continuing to investigate ways to reduce this amount even further.

KEY RESEARCH ACCOMPLISHMENTS:

- A web page portal that introduces users to our database has been created, and contains information about the capabilities of the database and how to perform searches.

- A biocurated sequence database, which will hold all data for the project, has been created and will evolve with the project as we identify new capabilities.

- A desktop database has been developed with commercial software (Filemaker Pro) to serve as a holding site for data. This database serves as a quality control step that allows confirmation and double-checking of data prior to entry into the public database.

- Interaction has begun with our taxonomy advisory committee to implement proper nomenclature. Fungi with nomenclatural inconsistencies have already been identified.

- A universal DNA extraction strategy has been developed that utilizes a simple extraction protocol and yields greater than 90% success on first pass.

- Universal primers capable of amplifying all fungi have been identified through empirical testing and genome sequence searching.

- Type cultures are being collected and cost-saving strategies have been implemented that include identifying free strains or the least expensive strain from multiple collections.

- New sequencing strategies have been implemented that reduce the cost of reagents.

REPORTABLE OUTCOMES:

1) A graduate student has been added to the laboratory and will be supported by the grant. Her dissertation will incorporate most of the proposal aims.

2) A number of clinical identifications have already been done. One of these was written up as a case report (Catheter-Related Fungemia due to *Candida thermophila*, J. Clin. Mic. 44:3035-6, see appendix) with acknowledgement of PRMRP funding.

3) We have also used our database to identify two fungi from clinical specimens from Brooke Army Medical Center, Fort Sam Houston, Texas. As an extension of this work, we have identified a collaborator (COL. Duane R. Hospenthal, Chief, Infectious Disease Service Brooke Army Medical Center, Fort Sam Houston, Texas) for a potential NIH proposal dealing with fungal identification from burn patients.

4) I am a Co-investigator on an NIH proposal entitled “Detection and significance of antifungal resistance in oropharyngeal candidiasis”. Funding was awarded 07/06 and runs until 06/11. Our role on the project will be to use our sequence database (developed from the PRMRP award) for identification of fungi isolated from AIDS patients.

CONCLUSION: Throughout the first year of the project, each of the tasks that were proposed to be worked on in the first 12 months is on schedule, or ahead of schedule. Since we are developing a universal technique that will be applicable to all fungi, a number of methods needed to be standardized so that they could be performed and accomplished independent of the species of fungus. Our data to date suggests that this goal has been achieved, as we have developed a simple universal extraction method and a universal PCR method, which most importantly, uses a single extraction reagent and the same set of primers for each isolate. Databases have been set up

and data are now being added. A web portal has been created to allow access to search algorithms for the database. Preliminary identifications using our methods have been successful and already have resulted in a publication, as well as a successful collaboration on an NIH grant. Type cultures and clinical cultures are being added to our collections, and their sequences are being added to the databases. The framework, from database entries to sequence recovery, has been established and will be continually ramped up as we add sequences at a faster rate. We will also continue to improve our methods, such as a filter based DNA extraction (for easy transport) and sequencing (for cost reduction), in order to make the entire process as efficient as possible.

REFERENCES:

1. McGinnis, S., and T. L. Madden. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools Nucleic Acids Res. 32:W20-5.

2. White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. 1990. Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics., p. 315-322. *In* N. Innis, D. Gelfand, J. Sninsky, and T. C. White (ed.), PCR-Protocols and Applications-A Laboratory Manual. Academic Press, New York).

3. Kurtzman, C. P., and C. J. Robnett. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216-23. Peterson, S. W., and C. P. Kurtzman. 1990. Phylogenetic relationships among species of the genus *Issatchenkia Kudriavzev*. Antonie Van Leeuwenhoek 58:235-40).

APPENDICES:

1) Publication: Bar-Meir M , Sutton DA , Wickes B , Kurtzman CP , Goldman S , Zheng X. 2006. Catheter-related fungemia due to *Candida thermophila*. J Clin Microbiol. 44:3035-6.

SUPPORTING DATA: N/A

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2006, p. 3035–3036 Vol. 44, No. 8
0095-1137/06/$08.00+0 doi:10.1128/JCM.00620-06

Catheter-Related Fungemia Due to *Candida thermophila*

Maskit Bar-Meir,[1] Deanna A. Sutton,[2] Brian Wickes,[2] Cletus P. Kurtzman,[3] Stewart Goldman,[1] and Xiaotian Zheng[1]*

Children's Memorial Hospital, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60614[1]; University of Texas Health Sciences Center, San Antonio, Texas 78229[2]; and National Center for Agricultural Utilization Research, ARS, U.S. Department of Agriculture, Peoria, Illinois 61604[3]

Received 22 March 2006/Returned for modification 15 May 2006/Accepted 31 May 2006

We report a case of bloodstream infection caused by *Candida thermophila*, a yeast not previously associated with human disease. The infection occurred in a 13-year-old boy with medulloblastoma who presented with 1 day of fever. Multiple blood cultures were positive for yeast. Removal of the catheter resulted in prompt resolution of the fever and sterilization of the blood cultures. The species was identified by sequencing domains 1 and 2 of the large subunit rRNA gene. Antifungal susceptibility testing was also performed.

CASE REPORT

A 13-year-old boy with medulloblastoma presented to the emergency department because of a 1-day history of fever up to 39.3°C, decreased oral intake, and increased fatigue. The tumor was diagnosed 14 months prior to his presentation. The patient was treated according to the Children's Oncology Group A-9961 protocol with surgical resection followed by reduced-dose craniospinal irradiation and alternate cycles of cisplatin, vincristine, and cyclophosphamide. The last cycle was given 3 weeks prior to his presentation. A central venous catheter, in place for a year, was used for administration of chemotherapy and hyperalimentation. The patient also received *Pneumocystis jiroveci* pneumonia prophylaxis with trimethoprim-sulfamethoxazole (160 mg of the trimethoprim component twice daily for three consecutive days each week).

Physical examination showed a febrile but otherwise well-appearing boy. The central line site showed no signs of infection or inflammation. Total white blood cell count was 4,300/mm^3 with 3,400 neutrophils/mm^3 and 560 band forms/mm^3, hemoglobin was 8.1 g/dl, and platelets were 59,000/mm^3. Findings on a chest radiograph were normal. A blood culture was drawn from the central line; the patient was given a dose of ceftriaxone and was sent home. The blood culture grew yeast after 24 h, and the patient was called and admitted to the hospital. At that time he was still well appearing and afebrile. An additional set of central and peripheral blood cultures was obtained, and administration of intravenous liposomal amphotericin (AmBisome) at 200 mg (5 mg/kg of body weight) once a day was begun. Altogether, eight sets of standard blood cultures (BACTEC Peds plus/F and standard anaerobic/F for each) and four sets of fungal blood cultures (ISOLATOR 1.5; Wampole Laboratories) were drawn over a 5-day period, and nine (five of the standard blood culture bottles and all fungal cultures) grew yeast. Sterilization of the blood was achieved only following removal of the central venous catheter on the fifth day of the antifungal therapy. The patient completed 6 weeks of liposomal amphotericin therapy and recovered without complications.

Laboratory findings. The yeast isolate from the patient grew after 24 to 48 h of incubation at 37°C. The colonies were moist and white in color. The germ tube test was negative. No hyphae or pseudohyphae were observed. The isolate was evaluated by the Microscan Walkaway system with a yeast identification plate (Dade Behring) and the API 20C AUX system (bioMerieux). Both gave an identification of *Hansenula polymorpha*. When biochemical reactions were run independently of the rapid systems, the isolate was negative for urease and positive for nitrate and glucose. The yeast grew at 37 and 42°C but not at 50°C.

Since the yeast could not be identified satisfactorily with the Microscan and API identification systems, DNA sequencing was conducted to provide identification. The isolate was identified as *Candida thermophila* (9) from its unique DNA sequence in domains 1 and 2 (D1/D2) of the large subunit rRNA gene by the National Center for Agricultural Utilization Research in Peoria and by the University of Texas Health Sciences Center in San Antonio. As described earlier (5, 6), genomic DNA was extracted from the yeast cells and combined with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) in a PCR. The resulting D1/D2 amplicon of ca. 600 nucleotides in length was purified, and both DNA strands were sequenced using primers NL-1 and NL-4 and an ABI (Applied Biosystems) automated DNA sequencer. The GenBank accession number for this sequence is DQ402185. The sequence of the isolate differed from that of *Candida thermophila* (GenBank accession AF283568) by one nucleotide. Other phylogenetically closely related organisms included *Pichia salicis* (GenBank accession AF403148; 99% identity), a presently undescribed species, and *Pichia angusta* (GenBank accession U75524; 98% identity) (4, 6). Our isolate has been deposited with the ARS Culture Collection as NRRL Y-27863 and with the American Type Culture Collection (ATCC MYA-3665).

Antifungal drug susceptibility testing was performed by the broth microdilution method based on the CLSI (formerly NCCLS) guidelines (8). Briefly, RPMI medium was used. The inoculation size was 5×10^4 CFU. MICs were read

* Corresponding author. Mailing address: Children's Memorial Hospital, Northwestern University Feinberg School of Medicine, 2300 Children's Plaza, Box 53, Chicago, IL 60614. Phone: (773) 880-6910. Fax: (773) 880-4687. E-mail: x-zheng@northwestern.edu.

TABLE 1. MIC and minimum fungicidal concentration results

Drug	MIC (μg/ml) at 24 h	MIC (μg/ml) at 48 h	MFC[a] (μg/ml)
Amphotericin	≤0.03	0.125	0.25
Caspofungin	0.12	0.5	1
Flucytosine	0.12	2	≥128
Fluconazole	1	2	16
Itraconazole	0.06	0.25	0.5
Ketoconazole	≤0.03	0.125	1
Voriconazole	≤0.03	≤0.03	≤0.03

[a] MFC, minimum fungicidal concentration.

at 24 and 48 h by comparing the turbidity of test wells to that of the untreated controls. A change in turbidity equal to or greater than 90% compared to drug-free control results was used to establish MIC breakpoints. The minimal fungicidal concentration results were obtained by recording colony counts on plates. Results are summarized in Table 1.

Discussion. Invasive candidiasis is an important cause of morbidity and mortality in chronically or critically ill patients (2, 3). Infections caused by *Candida* species are the fourth most common cause of nosocomial bloodstream infection in the United States (1, 11), with species other than *Candida albicans* emerging as pathogens. The non-*C. albicans* yeasts are often associated with resistance to antifungal azoles and with higher mortality. We describe the first reported case of *Candida thermophila* causing a human infection.

C. thermophila was described as a thermophilic soil yeast capable of growth at 50°C (9). Although the current isolate did not grow at 50°C, it did grow well at 37 and 42°C. Since it is difficult to identify this species with either commercial or conventional biochemical assays, this characteristic of growth in elevated temperature can be an indication for further analysis such as rRNA gene sequencing. Identification of yeasts from the large subunit rRNA gene D1/D2 sequence comparisons has been highly reliable. Strains of the same species ordinarily show only 0 to 3 nucleotide differences (6), but a few exceptions to this pattern have been found. For example, *Candida guilliermondii* and *Candida fermentati* differ by 3 nucleotides in large-subunit D1/D2 but show only 40% relatedness when compared by nuclear DNA reassociation (10). Consequently, these two taxa are closely related but not conspecific. The current isolate was identified as *C. thermophila* based on its close genetic similarity to the type strain of this species. The single nucleotide difference with the type strain has been interpreted as intraspecies strain variation. Gene sequence analysis has been successfully used for the identification of pathogenic fungi in addition to analysis of morphological and biochemical characteristics (7).

Although this is the first reported case of *C. thermophila* causing candidemia in a human, this may not be the first case of invasive disease due to this recently identified yeast, because identification and differentiation of yeasts on the basis of morphological and biochemical characteristics can be difficult. Therefore, the incidence and prevalence of this organism and its pathogenic role might be underestimated.

Many *Candida* species causing invasive infections have been non-*C. albicans* yeasts such as *C. krusei* and *C. glabrata*. These species can be inherently (primarily) or secondarily resistant to fluconazole and may be more difficult to treat. The isolate from our patient was susceptible to all antifungals in vitro, and the patient was treated successfully with liposomal amphotericin, although fluconazole might have been as effective.

In summary, as the population of immunocompromised hosts grows, organisms previously not considered as pathogens might cause invasive disease. *C. thermophila* should be added to the long list of yeasts that can cause bloodstream infections in the immunocompromised or critically ill patient.

Nucelotide sequence accession number. The sequence of the D1/D2 amplicon described in this study has been deposited under GenBank accession no. DQ402185.

We thank Annette W. Fothergill, Dora I. McCarthy, Mollyn Valentin, and William Kabat for technical assistance. We also thank Stanford Shulman for helpful discussion and review of the manuscript.

B.W. is supported by grant PR054228, from the U.S. Army Medical Research and Materiel Command, Office of Congressionally Directed Medical Research Programs.

REFERENCES

1. **Banerjee, S. N., T. G. Emori, D. H. Culver, R. P. Gaynes, W. R. Jarvis, T. Horan, J. R. Edwards, J. Tolson, T. Henderson, and W. J. Martone.** 1991. Secular trends in nosocomial primary bloodstream infections in the United States, 1980–1989. National Nosocomial Infections Surveillance System. Am. J. Med. **91**(3B)**:**86S–89S.
2. **Hostetter, M. K.** 1996. New insights into candidal infections. Adv. Pediatr. **43:**209–230.
3. **Kossoff, E. H., E. S. Buescher, and M. G. Karlowicz.** 1998. Candidemia in a neonatal intensive care unit: trends during fifteen years and clinical features of 111 cases. Pediatr. Infect. Dis. J. **17:**504–508.
4. **Kurtzman, C. P.** 1998. *Pichia*, E. C. Hansen emend. Kurtzman, p. 273–352. *In* C. P. Kurtzman and J. W. Fell (ed.), The yeasts, a taxonomic study, 4th ed. Elsevier Science B. V., Amsterdam, The Netherlands.
5. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35:**1216–1223.
6. **Kurtzman, C. P., and C. J. Robnett.** 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Leeuwenhoek **73:**331–371.
7. **McGinnis, M. R., and G. B. Nunn.** 2004. Sequence-based fungal identification and classification. *In* D. Persing, F. Tenover, Y. Tang, J. Versalovic, T. White, and E. Unger (ed.), Molecular microbiology: diagnostic principles and practices. ASM Press, Washington, D.C.
8. **NCCLS.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard. M27-A2. NCCLS, Wayne, Pa.
9. **Shin, K-S., Y. K. Shin, J.-H. Yoon, and Y.-H. Park.** 2001. *Candida thermophila* sp. nov., a novel thermophilic yeast isolated from soil. Int. J. Syst. Evol. Microbiol. **51:**2167–2170.
10. **Vaughan-Martini, A., C. P. Kurtzman, S. A. Meyer, and E. B. O'Neill.** 2005. Two new species in the *Pichia guilliermondii* clade: *Pichia caribbica* sp. nov., the ascosporic state of *Candida fermentans*, and *Candida carpophila*. comb. nov. FEMS Yeast Res. **5:**463–469.
11. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. **39:**309–317.